

## **CD1d-dependent natural killer T cells attenuate angiotensin II-induced cardiac remodeling via IL-10 signaling in mice**

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**Aims** Cd1d is a member of the cluster of differentiation 1 (CD1) family of glycoproteins expressed on the surface of various antigen-presenting cells, which is recognized by natural killer T (NKT) cells. CD1d-dependent NKT cells play an important role in immune-mediated diseases; but the role of these cells in regulating cardiac remodeling remains unknown. **Methods and Results** Cardiac remodeling was induced by angiotensin (Ang) II infusion for 2 weeks. Ang II-induced increase in hypertension, cardiac performance, hypertrophy and fibrosis, inflammatory response and activation of the NF- $\kappa$ B and TGF- $\beta$ 1/Smad2/3 pathways was significantly aggravated in CD1d knockout (CD1d<sup>ko</sup>) mice compared with wild type (WT) mice, but these effects were markedly abrogated in WT mice treated with  $\alpha$ -galactosylceramide ( $\alpha$ GC), a specific activator of NKT cells. Adoptive transfer of CD1d<sup>ko</sup> bone marrow cells to WT mice further confirmed the deleterious effect of CD1d knockout. Moreover, IL-10 expression was significantly decreased in CD1d<sup>ko</sup> hearts but increased in  $\alpha$ GC-treated mice. Co-culture experiments revealed that CD1d<sup>ko</sup> dendritic cells (DCs) significantly reduced IL-10 mRNA expression from NKT cells. Administration of recombinant murine IL-10 to CD1d<sup>ko</sup> mice improved hypertension, cardiac performance and adverse cardiac remodeling induced by Ang II, and its cardioprotective effect was possibly associated with activation of STAT3, and inhibition of the TGF- $\beta$ 1 and NF- $\kappa$ B pathways. **Conclusion** These findings revealed a previously undefined role for CD1d-dependent NKT cells in Ang II-induced cardiac remodeling, hence activation of NKT cells may be a novel therapeutic target for hypertensive cardiac disease.

**Keywords:** CD1d • natural killer T cells • angiotensin II • cardiac remodeling • IL-10

## 1. Introduction

Cardiac remodeling is the main ways by which the heart responds to mechanical and neurohormonal stimuli such as angiotensin II (Ang II)<sup>1</sup>. There are many common features during this process, including increased cardiomyocyte size, extracellular matrix deposition, and immune cell infiltration and activation<sup>1</sup>. Cardiac hypertrophy initially represents an adaptive response of the myocardium, ultimately leading to left ventricular dilatation and heart failure<sup>1</sup>. It has been well demonstrated that immune cells respond much earlier to cardiac injury, and activation of immune cells, including T lymphocytes and macrophages, leads to the secretion of many cytokines and growth factors, which play a critical role in the development of Ang II- and pressure overload-induced cardiac hypertrophy, fibrosis and heart failure<sup>2-5</sup>.

Natural killer T (NKT) cells are a unique T lymphocyte sublineage, which is characterized by co-expression of NK receptors and invariant T cell receptors (TCRs)<sup>6</sup>. NKT cells are reactive to the class I antigen-presenting molecule, CD1d, which is predominantly expressed on antigen-presenting cells<sup>6, 7</sup>. Based on differences in TCR characteristics, CD1d-dependent NKT cells are mainly divided into type I and type II NKT cells. The majority of NKT cells in mice comprise type I cells (invariant NKT cells, iNKT), which recognize the synthetic glycolipid,  $\alpha$ -galactosylceramide ( $\alpha$ -GC)<sup>6</sup>. Importantly, upon TCR activation, NKT cells are capable of quickly producing a wide array of growth factors, cytokines and chemokines, including pro-inflammatory [interferon  $\gamma$  (IFN- $\gamma$ ), interleukin-2 (IL-2), tumor necrosis factor (TNF)- $\alpha$ ] and/or anti-inflammatory cytokines (IL-4, IL-5, IL-10, IL-13)<sup>8</sup>. Previous studies have reported that CD1-deficient mice lack NKT cells and show reduced IL-4, IL-12, IFN- $\gamma$  level and IgE production<sup>9, 10, 11</sup>. CD1d-deficient mice are more susceptible to some viruses, bacteria and protozoa, and have a lower survival rate after influenza virus infection<sup>12</sup>. Recently, a study showed that mice lacking CD1d-expressing B cells have reduced iNKT cell activation and IFN- $\gamma$  production, and develop exacerbated arthritis compared with wild-type (WT) mice<sup>13</sup>. Increasing evidence demonstrated that NKT cells play a bidirectional role in the regulation of the immune response associated with a broad range of diseases. Activation of NKT cells promotes the development of inflammatory bowel disease, atherosclerosis and kidney injury through the Th1 immune response<sup>14-17</sup>. Conversely, NKT cells have a protective role against type 1 diabetes, allergic encephalomyelitis, rheumatoid arthritis and ischemic heart diseases<sup>18-22</sup>. Interestingly, the infiltration of iNKT cells was increased during the

early phase in the noninfarcted left ventricle after ischemia/reperfusion (I/R) or myocardial infarction (MI), and  $\alpha$ -GalCer further enhanced them<sup>20, 21</sup>. Ang II treatment also stimulates splenic NKT cells activation in low-density lipoprotein receptor (LDLr)- knockout mice<sup>23</sup>. However, no previous studies have examined the change in NKT cells and their role in Ang II-induced cardiac remodeling.

In this study, we examined cardiac remodeling in CD1d knockout (CD1dko) mice,  $\alpha$ GC-treated mice and bone marrow (BM)-transplanted CD1dko chimeric mice after Ang II infusion. Our results showed that Ang II-induced hypertension, cardiac remodeling and inflammation were aggravated in CD1dko and chimeric mice, whereas this effect was attenuated by  $\alpha$ GC-treated mice. Co-culture experiments revealed that CD1dko dendritic cells (DCs) significantly reduced IL-10 mRNA expression from NKT cells. Moreover, administration of IL-10 to Cd1dko mice markedly reversed Ang II-induced hypertension and adverse cardiac remodeling. This effect was associated with activation of STAT3 and inhibition of the TGF- $\beta$ 1 and NF- $\kappa$ B pathways. Thus, our results provide novel evidence supporting that activation of CD1d-dependent NKT cells have a protective role against Ang II-induced cardiac remodeling.

## **2. Methods**

Detailed description of the Methods is available in the online-only Data Supplement.

### **2.1 Animals**

The Cd1dko mice [B6(C)-Cd1d1tm1.2Aben/J] carrying a targeted knockout of the Cd1d1 (CD1d1 antigen) gene (Exons 2-6) were purchased from the Jackson Laboratory. Cardiac remodeling was induced in 10- to 12-week-old male Cd1dko mice and in matched C57BL/6 WT mice by subcutaneous infusion of 1000 ng/kg/min Ang II (Sigma-Aldrich, St Louis, MO, USA) for 2 weeks using osmotic mini-pumps (Alzet MODEL 1007D; DURECT, Cupertino, CA, USA) as described previously<sup>24</sup>. Saline infusion was used as the control. WT mice were intraperitoneally injected with 0.1  $\mu$ g/g  $\alpha$ GC (Funakoshi, Bunkyo-ku, Japan) on day 0, 1, 3, 5, 7, 9, 11 and 13. For IL-10 administration, Cd1dko mice were subcutaneously injected with 50  $\mu$ g/kg mouse recombinant IL-10 (PeproTech, Rocky Hill, USA) on day 0, 1, 3, 5, 7, 9, 11 and 13<sup>21</sup>. All mice were intraperitoneally anaesthetized with 0.25 mg/g tribromoethanol. All animal experiments performed in this study adhered to the protocols approved by the Institutional Animal Care and Use

Committee of Capital Medical University. All animal studies were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

## 2.2 Generation of Chimeric Mice

Chimeric mice were generated as described previously<sup>25</sup>. BM cells were collected from C57BL/6 WT and Cd1dko mice by flushing femurs and tibiae with RPMI-1640 medium. After recipient mice (8-week-old) were lethally irradiated (8.5 Gy), they received  $5 \times 10^6$  BM cells from C57BL/6 WT or Cd1dko mice. To verify the successful reconstitution of the BM in transplanted mice, we genotyped WT or CD1dko mice transplanted with CD1dko BM cells (CD1dko→WT) or WT BM cells (WT→CD1dko), respectively. Using the protocols provided by the The Jackson Laboratory, PCR analysis showed that the product in the BM and blood was 391 bp (WT), and the product in the heart and liver was ~330 bp (mutant) after WT→CD1dko. In contrast, the product in the BM and blood was ~330 bp (mutant), and the product in the heart and liver was 391bp (WT) after CD1dko→WT. These mice were kept in individually ventilated cages and were given acidified, antibiotic water and sterilized food. After 4 weeks of transplantation, these mice were infused with Ang II for 2 weeks. Levofloxacin (5%) and fluconazol (0.5%) were used in the BM transfer experiment in all groups.

## 2.3 Co-culture experiments

Dendritic cells (DCs) ( $5 \times 10^6$ ) isolated from WT and CD1dko mice were co-cultured with NKT cells ( $1 \times 10^5$ ) from WT mice. After 24 hours of co-culturing with or without Ang II stimulation, the supernatant was collected and centrifuged, and then RNA was extracted from NKT cells and the mRNA level of IL-10 was examined by qPCR analysis. Isolation of primary cardiac fibroblasts (CFs) was performed as described previously<sup>4</sup>. CFs ( $5 \times 10^6$ ) were co-cultured with NKT cells ( $1 \times 10^5$ ) from WT or CD1dko mice with or without Ang II stimulation for 24 hours. Protein was extracted from CFs to examine the protein levels of  $\alpha$ -SMA, TGF- $\beta$ 1, p-Smad2/3 and Smad2/3 by western blot analysis.

## 2.4 Statistical analysis

All values in the text and figures are expressed as mean  $\pm$  SEM. Comparisons between groups of

mice or treatments were conducted using one-way ANOVA or two-way ANOVA followed by either the post-hoc test (Student-Newman-Keuls) or Bonferroni tests when the ANOVA F values were less than 0.05.

### **3. Results**

#### **3.1 CD1d deficiency accelerates Ang II-Induced cardiac remodeling in mice**

To determine the role of the CD1d-dependent NKT cells in modulating Ang II-induced remodeling, we first assessed the effect of depleting these cells in WT and CD1dko mice. Flow cytometry analysis revealed that Ang II infusion for 2 weeks significantly increased the number of NKT cells in WT hearts, which was fully attenuated in CD1dko mice (Supplementary material, Figure 1A). The systolic blood pressure (SBP) was elevated in the Ang II-treated WT and CD1dko mice compared with the saline-treated groups, and the SBP was higher in CD1dko mice than in WT mice (Supplementary material, Figure 2A). Moreover, cardiac dysfunction as reflected by increased left ventricular (LV) ejection fraction (EF%), fractional shortening (FS%) and reduced E/A ratio, cardiac hypertrophy as indicated by increased LV wall thickness, heart weight/body weight (HW/BW) and HW/tibia length (TL) ratios, cross-sectional area of myocytes and the mRNA levels of atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP) in the heart were observed in Ang II-treated WT and CD1dKo mice compared with saline groups, and these effects were further enhanced in CD1dko mice after Ang II infusion (Figure 1A-C, Table S1).

We next examined the effect of Cd1d on cardiac fibrosis and TGF- $\beta$ 1 signaling. Ang II infusion markedly increased the interstitial and perivascular fibrosis, the mRNA expression of collagen I and collagen III, and the protein levels of TGF- $\beta$ 1 and phosphorylated Smad2/3 in the hearts of WT and CD1dko mice, which was further exacerbated in the CD1dko mice (Figure 1D-E). There was no significant difference in the parameters of cardiac remodeling and signaling mediators between the two groups after saline infusion (Figure 1A-E). Overall, these data clearly suggest that CD1dko mice are more susceptible to Ang II-induced hypertension and cardiac remodeling.

#### **3.2 Administration of an NKT cell activator attenuates Ang II-induced cardiac remodeling in mice**

To assess the causative role of CD1d-restricted NKT cells in the development of cardiac remodeling, we systemically co-treated WT mice with aGC (0.1 µg/g), a specific activator of NKT cells, and Ang II for 14 days. Administration of aGC to WT mice significantly increased the number of NKT cells in the heart tissue, but this increase was totally attenuated in CD1d<sup>ko</sup> mice (Supplementary material, Figure 1B). Moreover, the SBP, cardiac performance (EF% and FS%) and hypertrophy (increased LV wall thickness, the HW/BW and HW/TL ratios, the cross-sectional area of myocytes and the mRNA levels of ANF and BNP in the heart) were increased in Ang II-treated WT mice compared with saline-treated control groups, and these effects were reduced in WT mice co-treated with Ang II- and aGC (Supplementary material, Figure 2B and Figure 2A-C). In addition, aGC-treated WT mice exhibited a marked reduction of cardiac fibrosis, collagen I and collagen III expression and activation of TGF-β1 and Smad2/3 in the hearts compared with the vehicle control after Ang II infusion (Figure 2D-E). There was no statistical difference in cardiac performance and remodeling between the saline and aGC-treated mice at baseline (Figure 2A-E).

### **3.3 CD1d deficiency promotes Ang II-induced myocardial inflammation**

We next examined whether CD1d deletion influences myocardial inflammatory cell infiltration. Flow cytometry showed that Ang II infusion significantly increased infiltration of CD45<sup>+</sup> cells, CD11b<sup>+</sup>F4/80<sup>+</sup> macrophages, CD11b<sup>+</sup>Gr1<sup>+</sup> neutrophils and CD3<sup>+</sup>T cells in both WT and CD1d<sup>ko</sup> hearts compared with the saline-treated groups (Figure 3A). However, compared with the WT mice, the number of CD45<sup>+</sup> cells was lower and the number of CD11b<sup>+</sup>F4/80<sup>+</sup> macrophages was higher in CD1d<sup>ko</sup> mice after Ang II treatment (Figure 3A). Although the Ang II-induced infiltration of CD11b<sup>+</sup>Gr1<sup>+</sup> neutrophils and CD3<sup>+</sup>T cells was lower in CD1d<sup>ko</sup> hearts than in WT, there was no significant difference between two groups (Figure 3A). The number of these cells was similar between WT and CD1d<sup>ko</sup> hearts after saline infusion (Figure 3A). Moreover, the accumulation of the inflammatory cells, Mac-2-positive macrophages, the mRNA levels of IL-1β and TNF-α and p65 phosphorylation in the hearts were increased in WT and CD1d<sup>ko</sup> mice, and this increase was more obvious in CD1d<sup>ko</sup> mice (Figure 3B-D), however, it was markedly attenuated in aGC-treated mice compared with controls (Supplementary material, Figure 3A-C).

### **3.4 Selective deletion of CD1d in bone marrow-derived cells aggravates Ang II-induced**

### **cardiac remodeling and inflammation**

To specifically determine the role of myeloid cell CD1d expression in the development of cardiac remodeling, we generated chimeric mice by transplanting bone marrow (BM)-derived cells from CD1d<sup>ko</sup> and WT mice into lethally irradiated CD1d<sup>ko</sup> and WT mice. After 4 weeks of transplantation, these mice were infused with Ang II for additional 2 weeks. Compared with WT mice transplanted with WT BM (WT→WT), WT mice transplanted with CD1d<sup>ko</sup> BM (CD1d<sup>ko</sup>→WT) exhibited increased cardiac performance (EF% and FS%), hypertrophy (HW/BW and TL/BW ratios, myocyte cross-sectional area and expression of ANP and BNP in the heart), fibrosis (collagen deposition and expression), infiltration of Mac-2-positive macrophages and expression of IL-1 $\beta$  and TNF- $\alpha$  in the heart (Figure 4A-E). Similar effects were observed in CD1d<sup>ko</sup> mice transplanted with CD1d<sup>ko</sup> BM (CD1d<sup>ko</sup>→CD1d<sup>ko</sup>) (Figure 4A-E). In contrast, CD1d<sup>ko</sup> mice transplanted with WT BM (WT→CD1d<sup>ko</sup>) reversed these pathological features of cardiac functional alterations and remodeling compared with the CD1d<sup>ko</sup>→WT and CD1d<sup>ko</sup>→CD1d<sup>ko</sup> groups (Figure 4A-E). Together, these results demonstrate that myeloid cell-specific CD1d deletion augments Ang II-induced cardiac remodeling.

### **3.5 CD1d deficiency reduces IL-10 expression from NKT cells causing fibroblast differentiation**

The results in Figure 3C showed that CD1d deficiency increased Th1-type cytokines (IL-1 $\beta$  and TNF- $\alpha$ ) in Ang II-treated hearts, we then tested whether CD1d deficiency reduced Th2-type cytokines (IL-10 and IL-4) in this model. qPCR analysis revealed that Ang II infusion upregulated the expression of IL-10 and IL-4 in the hearts of WT mice compared with saline-treated control. This effect was significantly reduced in CD1d<sup>ko</sup> mice (Figure 5A and Supplementary material, Figure 4A), but was enhanced in  $\alpha$ GC-treated hearts after Ang II infusion (Figure 5B and Supplementary material, Figure 4B). The change of IL-10 protein level in CD1d<sup>ko</sup> hearts or  $\alpha$ GC-treated WT hearts was further verified by ELISA assay (Figure 5C and 5D).

To determine which cells could produce IL-10, we measured IL-10 mRNA level in DCs or NKT cells isolated from WT and Cd1d<sup>ko</sup> mice, respectively. qPCR analysis showed that IL-10 mRNA expression was slightly increased in Ang II-treated DCs or NKT cells, and there was no significant difference between WT and Cd1d<sup>ko</sup> mice (Figure 5E). We next test whether CD1d<sup>+</sup>



DCs mediated the mRNA expression of IL-10 from NKT cells. Ang II treatment reduced the Cd1d mRNA level in DCs level compared with saline control (Supplementary material, Figure 4C). Furthermore, co-culture of NKT cells with CD1dko DCs significantly reduced IL-10 mRNA level in NKT cells compared with NKT cells co-cultured with WT DCs after Ang II treatment (Figure 5F), indicating that IL-10 is mainly produced from NKT cells via CD1d<sup>+</sup> DCs, and may be critical in this model.

We next examined the regulatory properties of NKT cells in activation of cardiac fibroblasts (CFs) *in vitro*. NKT cells isolated from WT or CD1dko mice were co-cultured with WT CFs. Immunoblotting analysis indicated that the protein levels of  $\alpha$ -SMA (a marker for myofibroblast differentiation), TGF- $\beta$ 1 and p-Smad2/3 were markedly upregulated in CFs co-cultured with WT or CD1dko NKT cells compared with saline controls, and this increase was enhanced in CFs co-cultured CD1dko NKT cells after Ang II stimulation (Figure 5G). These results indicate that CD1dko NKT cells are capable of promoting myofibroblast activation.

### **3.6 Administration of recombinant IL-10 improves cardiac remodeling in CD1dko mice**

We next tested whether IL-10 can prevent Ang II-induced hypertension and cardiac remodeling in WT and CD1dko mice. After Ang II treatment, IgG-treated CD1dko mice showed increased SBP, cardiac performance (EF% and FS%), hypertrophy (HW/BW and HW/TL ratios, myocyte cross-sectional area) and fibrosis (collagen deposition), infiltration of Mac-2-positive macrophages and expression of ANF, BNP,  $\beta$ -myosin heavy chain ( $\beta$ -MHC), collagen I and collagen III compared with IgG-treated WT mice (Figure 6A-E and Supplementary material, Figure 5 and Figure 6A-B). However, systemic administration of mouse recombinant IL-10 (50  $\mu$ g/kg) significantly reversed these pathological changes in WT and CD1dko mice compared with the IgG controls (Figure 6A-E and Supplementary material, Figure 5 and Figure 6A-B). Moreover, administration of IL-10 markedly increased the p-STAT3 level, but reduced the level of p65, TGF- $\beta$ 1 and  $\alpha$ -SMA protein levels in both WT and CD1dko mice compared with the IgG controls after Ang II infusion (Figure 6F). Together, these results suggest that IL-10 improves cardiac remodeling in CD1d-deficient mice, which was partially associated with activation of STAT3 and inhibition of the NF- $\kappa$ B and TGF- $\beta$ 1 pathways.

#### 4. Discussion

In this study, we evaluated the regulatory role of CD1d-dependent NKT cells in Ang II-induced cardiac remodeling. We showed that Cd1d deficiency significantly accelerated Ang II-induced hypertension, cardiac remodeling and inflammatory response, which were markedly improved by  $\alpha$ GC. Importantly, Cd1d-deficient DCs reduced IL-10 production by NKT cells. Administration of IL-10 to Cd1d<sup>ko</sup> mice significantly reversed the Ang II-induced hypertension and cardiac remodeling, possibly through activation of STAT3 and inhibition of the TGF- $\beta$ 1 and NF- $\kappa$ B signaling pathways.

Cardiac hypertrophy and fibrosis are pathological features of many cardiac diseases, including hypertension, cardiomyopathy and myocardial infarction. However, the mechanisms that regulate these diseases have not been fully elucidated. Recently, immune cells including monocytes, macrophages, dendritic cells (DCs) and T lymphocytes have been implicated in the development of hypertension, cardiac remodeling and heart failure<sup>5</sup>. Among them, T cells have been regarded as critical modulators in the onset of the cardiac proinflammatory response and remodeling. CD8<sup>+</sup> T cells promote macrophage activation, leading to cardiac injury upon Ang II infusion<sup>2</sup>. Deletion of IL-12p35 increases the CD4<sup>+</sup>T cell-dependent differentiation of M2 macrophages and the production of TGF- $\beta$ 1 to aggravate Ang II-induced cardiac fibrosis<sup>3</sup>.  $\gamma\delta$ T-cell-derived IL-17 also promotes Ang II-induced cardiac injury and fibrosis<sup>26</sup>. Moreover, our recent findings suggest that CXCL1-CXCR2 axis mediates monocyte activation, which induces hypertension thereby leading to cardiac remodeling<sup>25, 27, 28</sup>. In this study, we extended previous findings and clearly showed that CD1d-dependent NKT cells also exhibited a cardioprotective role. CD1d-deficiency exacerbates Ang II-induced elevation of SBP together with cardiac hypertrophy and fibrosis, whereas  $\alpha$ GC blunts this response (Figure 1 and 2, Figure S2), suggesting that Ang II-induced cardiac remodeling was partially secondary to BP after Cd1d deletion.

Proinflammatory cells-derived cytokines including IL-1  $\beta$ , IL-6, TNF- $\alpha$  and TGF- $\beta$ 1 have been implicated in the development of cardiac remodeling, most likely via downstream activation of many signaling mediators and transcription factors, such as NF- $\kappa$ B and Smad2/3<sup>27</sup>. Activation of NF- $\kappa$ B signaling significantly induces cardiac inflammation and hypertrophy<sup>29</sup>. Conversely, inhibition of NF- $\kappa$ B by ablation or various inhibitors markedly attenuated left ventricular hypertrophy induced by Ang II and chronic pressure overload<sup>30-32</sup>. TGF- $\beta$ -Smad signaling pathway

is considered a major contributor of cardiac fibrosis<sup>27</sup>. Dendritic cells (DCs) are highly specialized antigen-presenting cells with a unique ability to activate naive T lymphocytes. Our previous study suggested that Ang II can enhance the maturation and activation of DCs through activation of NF- $\kappa$ B, ERK and STAT1 signaling pathways<sup>33</sup>. Further, antigen-presenting molecule CD1d on DCs can activate NKT cells through their semi-invariant  $\alpha\beta$  T cell receptors (TCRs)<sup>34</sup>. The present study suggested that CD1d-expressing DCs regulated activation of NKT cells to produce multiple cytokines such as IL-1 $\beta$ , TNF- $\alpha$ , IL-4 and IL-10 after Ang II stimulation. Deletion of Cd1d upregulated IL-1 $\beta$  and TNF- $\alpha$  but reduced IL-4 and IL-10 accompanied with activation of the NF- $\kappa$ B pathways, and these effects were markedly reversed by  $\alpha$ GC (Figure 1 through 3, 5, Figure S3 and 4). Thus, our findings indicate that CD1d-dependent NKT cells exert a cardioprotective effect partially through reduction of proinflammatory cytokines and inhibition of the NF- $\kappa$ B and TGF- $\beta$ 1/Smad2/3 signaling cascades.

Increasing evidence suggests that NKT cells have opposite roles in various immune-based diseases through either Th1-cytokines or Th2-cytokines<sup>14-16,19-22, 35</sup>. A recent study has demonstrated that the balance of Th1/Th2 cytokines plays a critical role in cardiac remodeling and inflammation<sup>36</sup>. Consistent with the role of NKT cells in inducing Th2 cytokines in diabetes, rheumatoid arthritis and ischemic heart diseases<sup>19-22, 35</sup>, our results showed that NKT cell deficiency can cause a shift from Th2 to Th1 in Ang II-treated heart, while activation of NKT cells by  $\alpha$ GC had the opposite effect (Figure 5 and Figure S4). IL-10 is an anti-inflammatory cytokine that exerts different effects on hypertension through regulating multiple signaling pathways. IL-10 preserves endothelium-dependent vasorelaxation perhaps by reducing production of superoxide during diabetes<sup>37</sup>. Moreover, IL-10 limits Ang II-induced hypertension by inhibiting RhoA/Rho-kinase signaling in angiotensin II-infused mice. In contrast, IL-10 increases salt-sensitive hypertension and renal injury induced by Ang II through reduction of nitric oxide<sup>38</sup>. Here our results showed that IL-10 treatment markedly reduced Ang II-induced hypertension in both WT and CD1d $\text{ko}$  mice (Figure S5), which may contribute to attenuation of Ang II-cardiac remodeling (Figure 6). In addition to hypertension, IL-10 also has opposite role in regulation of cardiac remodeling via STAT3 and NF- $\kappa$ B. Administration of IL-10 significantly attenuates isoproterenol- or pressure overload-induced hypertrophic remodeling and improves heart function<sup>39</sup>. Moreover, IL-10 mediates the protection of  $\alpha$ GC against myocardial remodeling after

ischemia/reperfusion and post-infarction<sup>20, 21, 40</sup>. However, a recent study in mice and human showed that IL-10 exerts profibrotic deleterious actions in humans and mice. Cardiac macrophages produce IL-10, activate fibroblasts to collagen deposition, leading to impaired myocardial relaxation. Deletion of IL-10 in macrophages improves diastolic function<sup>41</sup>. In contrast to this study, our *in vitro* results revealed that CD1d-deficient DCs reduced IL-10 expression by NKT cells (Figure 5F), and CD1d<sup>ko</sup> NKT cells significantly promoted myofibroblast differentiation and TGF- $\beta$ 1/Smad2/3 signaling activation (Figure 5G), suggesting that IL-10 produced by NKT cells may be crucial in this model (Figure 5). Consistent with the results for the cardioprotective role of IL-10, the present data confirmed that IL-10 treatment markedly reduced Ang II-induced cardiac remodeling in both WT and CD1d<sup>ko</sup> mice, and this beneficial effect was partially associated with activation of STAT3 and inhibition of the TGF- $\beta$ 1 and NF- $\kappa$ B pathways (Figure 6). Overall, these data support the idea that activation of Cd1d-dependent NKT cells prevented Ang II-induced cardiac injury possibly through increased IL-10 production.

In conclusion, we demonstrated for the first time that activation of CD1d-dependent NKT cells plays a protective role in Ang II-induced cardiac remodeling and inflammation. NKT cells release IL-10, which inhibited cardiomyocyte hypertrophy and fibroblast differentiation through activation of STAT3 and inhibition of the TGF- $\beta$ 1 and NF- $\kappa$ B pathways. Thus, selective activation of NKT cells may represent a promising therapeutic approach for the treatment of hypertensive cardiac diseases. Further investigations in other animal models of cardiac hypertrophy and remodeling are needed to determine the clinical use of NKT cell activation as a pharmacological therapy.

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## Conflict of interest

None declared.

## References

1. Cohn JN, Ferrari R, Sharpe N. Cardiac remodeling--concepts and clinical implications: a consensus paper from an international forum on cardiac remodeling. Behalf of an International Forum on Cardiac Remodeling. *J Am Coll Cardiol* 2000;**35**:569-582.
2. Ma F, Feng J, Zhang C, Li Y, Qi G, Li H, Wu Y, Fu Y, Zhao Y, Chen H, Du J, Tang H. The requirement of CD8+ T cells to initiate and augment acute cardiac inflammatory response to high blood pressure. *J Immunol* 2014;**192**:3365-3373.
3. Li Y, Zhang C, Wu Y, Han Y, Cui W, Jia L, Cai L, Cheng J, Li H, Du J. Interleukin-12p35 deletion promotes CD4 T-cell-dependent macrophage differentiation and enhances angiotensin II-Induced cardiac fibrosis. *Arterioscler Thromb Vasc Biol* 2012;**32**:1662-1674.
4. Wang L, Li YL, Zhang CC, Cui W, Wang X, Xia Y, Du J, Li HH. Inhibition of Toll-like receptor 2 reduces cardiac fibrosis by attenuating macrophage-mediated inflammation. *Cardiovasc Res* 2014;**101**:383-392.
5. Frieler RA, Mortensen RM. Immune cell and other noncardiomyocyte regulation of cardiac hypertrophy and remodeling. *Circulation* 2015;**131**:1019-1030.
6. Skold M, Behar SM. Role of CD1d-restricted NKT cells in microbial immunity. *Infect Immun* 2003;**71**:5447-5455.
7. Godfrey DI, Kronenberg M. Going both ways: immune regulation via CD1d-dependent NKT cells. *The Journal of clinical investigation* 2004;**114**:1379-1388.
8. Wu L, Gabriel CL, Parekh VV, Van Kaer L. Invariant natural killer T cells: innate-like T cells with potent immunomodulatory activities. *Tissue Antigens* 2009;**73**:535-545.
9. Smiley ST, Kaplan MH, Grusby MJ. Immunoglobulin E production in the absence of interleukin-4-secreting CD1-dependent cells. *Science* 1997;**275**:977-979.
10. Exley MA, Bigley NJ, Cheng O, Shaulov A, Tahir SM, Carter QL, Garcia J, Wang C, Patten K, Stills HF, Alt FW, Snapper SB, Balk SP. Innate immune response to encephalomyocarditis virus infection mediated by CD1d. *Immunology* 2003;**110**:519-526.
11. Mendiratta SK, Martin WD, Hong S, Boesteanu A, Joyce S, Van Kaer L. CD1d1 mutant mice are deficient in natural T cells that promptly produce IL-4. *Immunity* 1997;**6**:469-477.
12. Ishikawa H, Tanaka K, Kutsukake E, Fukui T, Sasaki H, Hata A, Noda S, Matsumoto T. IFN-gamma production downstream of NKT cell activation in mice infected with influenza virus enhances the cytolytic activities of both NK cells and viral antigen-specific CD8+ T cells. *Virology* 2010;**407**:325-332.
13. Oleinika K, Rosser EC, Matei DE, Nistala K, Bosma A, Drozdov I, Mauri C. CD1d-dependent immune suppression mediated by regulatory B cells through modulations of iNKT cells. *Nat Commun* 2018;**9**:684.
14. Olszak T, Neves JF, Dowds CM, Baker K, Glickman J, Davidson NO, Lin CS, Jobin C, Brand S, Sotlar K, Wada K, Katayama K, Nakajima A, Mizuguchi H, Kawasaki K, Nagata K, Muller W, Snapper SB, Schreiber S, Kaser A, Zeissig S, Blumberg RS. Protective mucosal immunity mediated by epithelial CD1d and IL-10. *Nature* 2014;**509**:497-502.

15. Nakai Y, Iwabuchi K, Fujii S, Ishimori N, Dashtsoodol N, Watano K, Mishima T, Iwabuchi C, Tanaka S, Bezbradica JS, Nakayama T, Taniguchi M, Miyake S, Yamamura T, Kitabatake A, Joyce S, Van Kaer L, Onoe K. Natural killer T cells accelerate atherogenesis in mice. *Blood* 2004;**104**:2051-2059.
16. Rabb H. The promise of immune cell therapy for acute kidney injury. *The Journal of clinical investigation* 2012;**122**:3852-3854.
17. Getz GS, Reardon CA. Natural killer T cells in atherosclerosis. *Nat Rev Cardiol* 2017;**14**:304-314.
18. Van Kaer L, Wu L, Parekh VV. Natural killer T cells in multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis. *Immunology* 2015;**146**:1-10.
19. Miellot A, Zhu R, Diem S, Boissier MC, Herbelin A, Bessis N. Activation of invariant NK T cells protects against experimental rheumatoid arthritis by an IL-10-dependent pathway. *Eur J Immunol* 2005;**35**:3704-3713.
20. Homma T, Kinugawa S, Takahashi M, Sobirin MA, Saito A, Fukushima A, Suga T, Takada S, Kadoguchi T, Masaki Y, Furihata T, Taniguchi M, Nakayama T, Ishimori N, Iwabuchi K, Tsutsui H. Activation of invariant natural killer T cells by alpha-galactosylceramide ameliorates myocardial ischemia/reperfusion injury in mice. *J Mol Cell Cardiol* 2013;**62**:179-188.
21. Sobirin MA, Kinugawa S, Takahashi M, Fukushima A, Homma T, Ono T, Hirabayashi K, Suga T, Azalia P, Takada S, Taniguchi M, Nakayama T, Ishimori N, Iwabuchi K, Tsutsui H. Activation of natural killer T cells ameliorates postinfarct cardiac remodeling and failure in mice. *Circ Res* 2012;**111**:1037-1047.
22. Hong S, Wilson MT, Serizawa I, Wu L, Singh N, Naidenko OV, Miura T, Haba T, Scherer DC, Wei J, Kronenberg M, Koezuka Y, Van Kaer L. The natural killer T-cell ligand alpha-galactosylceramide prevents autoimmune diabetes in non-obese diabetic mice. *Nat Med* 2001;**7**:1052-1056.
23. van Puijvelde GHM, Foks AC, van Bochove RE, Bot I, Habets KLL, de Jager SC, Ter Borg MND, van Osch P, Boon L, Vos M, de Waard V, Kuiper J. CD1d deficiency inhibits the development of abdominal aortic aneurysms in LDL receptor deficient mice. *PLoS One* 2018;**13**:e0190962.
24. Wang X, Wang HX, Li YL, Zhang CC, Zhou CY, Wang L, Xia YL, Du J, Li HH. MicroRNA Let-7i negatively regulates cardiac inflammation and fibrosis. *Hypertension* 2015;**66**:776-785.
25. Wang L, Zhao XC, Cui W, Ma YQ, Ren HL, Zhou X, Fassett J, Yang YZ, Chen Y, Xia YL, Du J, Li HH. Genetic and Pharmacologic Inhibition of the Chemokine Receptor CXCR2 Prevents Experimental Hypertension and Vascular Dysfunction. *Circulation* 2016;**134**:1353-1368.
26. Li Y, Wu Y, Zhang C, Li P, Cui W, Hao J, Ma X, Yin Z, Du J. gammadeltaT Cell-derived interleukin-17A via an interleukin-1beta-dependent mechanism mediates cardiac injury and fibrosis in hypertension. *Hypertension* 2014;**64**:305-314.
27. Wang L, Zhang YL, Lin QY, Liu Y, Guan XM, Ma XL, Cao HJ, Liu Y, Bai J, Xia YL, Du J, Li HH. CXCL1-CXCR2 axis mediates angiotensin II-induced cardiac hypertrophy and remodelling through regulation of monocyte infiltration. *European heart journal* 2018;**39**:1818-1831.
28. Paradis P, Schiffrin EL. CXCL1-CXCR2 lead monocytes to the heart of the matter. *European heart journal* 2018;**39**:1832-1834.
29. Freund C, Schmidt-Ullrich R, Baurand A, Dunger S, Schneider W, Loser P, El-Jamali A, Dietz R, Scheidereit C, Bergmann MW. Requirement of nuclear factor-kappaB in angiotensin II- and

- isoproterenol-induced cardiac hypertrophy in vivo. *Circulation* 2005;**111**:2319-2325.
30. Zelarayan L, Renger A, Noack C, Zafiriou MP, Gehrke C, van der Nagel R, Dietz R, de Windt L, Bergmann MW. NF-kappaB activation is required for adaptive cardiac hypertrophy. *Cardiovascular research* 2009;**84**:416-424.
  31. Wang C, Li L, Zhang ZG, Fan D, Zhu Y, Wu LL. Globular adiponectin inhibits angiotensin II-induced nuclear factor kappaB activation through AMP-activated protein kinase in cardiac hypertrophy. *Journal of cellular physiology* 2010;**222**:149-155.
  32. Esposito G, Rapacciuolo A, Naga Prasad SV, Takaoka H, Thomas SA, Koch WJ, Rockman HA. Genetic alterations that inhibit in vivo pressure-overload hypertrophy prevent cardiac dysfunction despite increased wall stress. *Circulation* 2002;**105**:85-92.
  33. Chen C, Meng Y, Wang L, Wang HX, Tian C, Pang GD, Li HH, Du J. Ubiquitin-activating enzyme E1 inhibitor PYR41 attenuates angiotensin II-induced activation of dendritic cells via the IkappaBa/NF-kappaB and MKP1/ERK/STAT1 pathways. *Immunology* 2014;**142**:307-319.
  34. Alvarez D, Vollmann EH, von Andrian UH. Mechanisms and consequences of dendritic cell migration. *Immunity* 2008;**29**:325-342.
  35. Singh AK, Wilson MT, Hong S, Olivares-Villagomez D, Du C, Stanic AK, Joyce S, Sriram S, Koezuka Y, Van Kaer L. Natural killer T cell activation protects mice against experimental autoimmune encephalomyelitis. *The Journal of experimental medicine* 2001;**194**:1801-1811.
  36. Fujiu K, Wang J, Nagai R. Cardioprotective function of cardiac macrophages. *Cardiovascular research* 2014;**102**:232-239.
  37. Gunnett CA, Heistad DD, Faraci FM. Interleukin-10 protects nitric oxide-dependent relaxation during diabetes: role of superoxide. *Diabetes* 2002;**51**:1931-1937.
  38. Singh P, Castillo A, Islam MT, Majid DSA. Evidence for Prohypertensive, Proinflammatory Effect of Interleukin-10 During Chronic High Salt Intake in the Condition of Elevated Angiotensin II Level. *Hypertension* 2017;**70**:839-845.
  39. Verma SK, Krishnamurthy P, Barefield D, Singh N, Gupta R, Lambers E, Thal M, Mackie A, Hoxha E, Ramirez V, Qin G, Sadayappan S, Ghosh AK, Kishore R. Interleukin-10 treatment attenuates pressure overload-induced hypertrophic remodeling and improves heart function via signal transducers and activators of transcription 3-dependent inhibition of nuclear factor-kappaB. *Circulation* 2012;**126**:418-429.
  40. Jung M, Ma Y, Iyer RP, DeLeon-Pennell KY, Yabluchanskiy A, Garrett MR, Lindsey ML. IL-10 improves cardiac remodeling after myocardial infarction by stimulating M2 macrophage polarization and fibroblast activation. *Basic Res Cardiol* 2017;**112**:33.
  41. Hulsmans M, Sager HB, Roh JD, Valero-Munoz M, Houstis NE, Iwamoto Y, Sun Y, Wilson RM, Wojtkiewicz G, Tricot B, Osborne MT, Hung J, Vinegoni C, Naxerova K, Sosnovik DE, Zile MR, Bradshaw AD, Liao R, Tawakol A, Weissleder R, Rosenzweig A, Swirski FK, Sam F, Nahrendorf M. Cardiac macrophages promote diastolic dysfunction. *The Journal of experimental medicine* 2018;**215**:423-440.

### Figure legends:

Figure 1. CD1d deficiency accelerates Ang II-induced cardiac remodeling. (A) Echocardiography was performed on CD1dko or WT mice after 14 days of Ang II infusion (1000 ng/kg/min) (top). Quantification of EF% and FS% (bottom; n=10 per group). (B) Representative H&E staining of heart sections (top). Scale bar: 2 mm. The ratios of the heart weight/body weight (HW/BW) and heart weight/tibia length (HW/TL) (bottom; n=10 per group). (C) Representative wheat germ agglutinin (WGA) staining of heart sections (left) and quantification of the cross-sectional area of myocytes (middle; n=6 per group). Scale bar: 50  $\mu$ m. qPCR analysis of the hypertrophic markers ANF and BNP mRNA expression in wild-type (WT) and CD1dko hearts after saline or Ang II infusion (right; n=6 per group). (D) Representative Masson's trichrome staining in heart sections of each group (left) and quantification of collagen deposition (middle; n=6 per group). Scale bar: 100  $\mu$ m. qPCR analysis of collagen I and collagen III mRNA expression in hearts (right; n=6 per group). (E) Western blot analysis for the protein expression of TGF- $\beta$ 1, p-Smad2/3 and Smad2/3 (left) and quantification of the protein bands (right; n=5 per group). Data are expressed as mean  $\pm$  SEM. \* $P$ <0.05, \*\* $P$ <0.01 vs saline group; # $P$ <0.05, ## $P$ <0.01 vs Ang II group.

Figure 2. aGC alleviates Ang II-induced cardiac remodeling. (A) Echocardiography was performed in wild type (WT) mice after 14 days of aGC administration (top). Quantification of EF% and FS% (bottom; n=10 per group). (B) Representative H&E staining of heart sections (top), Scale bar: 2 mm, and the ratios of heart weight/body weight (HW/BW) and heart weight/tibial length (HW/TL) (bottom; n=10 per group). (C) Representative wheat germ agglutinin (WGA) staining of heart sections (left) and quantification of the cross-sectional area of myocytes (middle; n=6 per group). Scale bar: 50  $\mu$ m. qPCR analysis of hypertrophic markers ANF and BNP mRNA expression in hearts (right; n=6 per group). (D) Representative Masson's trichrome staining of the heart sections of each group (left) and quantification of collagen deposition (middle; n=6 per group). Scale bar: 100  $\mu$ m. qPCR analysis of collagen I and collagen III mRNA expression in the hearts (right; n=6 per group). (E) Western blot analysis for the protein expression of TGF $\beta$ , p-Smad2/3 and Smad2/3 (left). Quantification of the protein bands (right; n=5 per group). Data are expressed as mean  $\pm$  SEM. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 vs vehicle groups. # $P$ <0.05, ## $P$ <0.01 vs Ang II group.



Figure 3. CD1d deficiency aggravates Ang II-induced cardiac inflammation and activation of NF- $\kappa$ B in the heart tissues. (A) Flow cytometry analysis of CD45<sup>+</sup> leukocytes, CD45<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> macrophages, CD45<sup>+</sup>CD11b<sup>+</sup>Gr-1<sup>+</sup> neutrophils and CD45<sup>+</sup>CD3<sup>+</sup> T lymphocytes in the hearts (n=4 per group). (B) Representative H&E and immunohistochemical staining of Mac-2 in heart sections from wild-type (WT) and CD1dKO mice (left). Scale bar: 20  $\mu$ m; Quantification of Mac-2-positive cells (right; n=6 per group). (C) qPCR analysis of IL-1 $\beta$  and TNF- $\alpha$  mRNA expression in the hearts (n=6 per group). (D) Immunoblotting analysis for the protein expression of p-p65 and p65 (top). Quantification of protein bands (bottom; n=5 per group). Data are expressed as mean  $\pm$  SEM. \*P < 0.05, \*\*P < 0.01 vs saline or vehicle group. #P < 0.05, ##P < 0.01 vs Ang II group.

Figure 4. Bone marrow-derived CD1dKO cells exacerbate cardiac remodeling induced by Ang II infusion. (A) Echocardiography was performed on wild-type (WT) mice after bone marrow (BM) transplantation (top). Quantification of EF% and FS% (bottom; n=10 per group). (B) Representative H&E staining of heart sections (top). Scale bar: 2 mm. The ratios of heart weight/body weight (HW/BW) and heart weight/tibial length (HW/TL) were calculated after BM transplantation (bottom; n=10 per group). (C) Representative wheat germ agglutinin (WGA) staining of the heart sections (left) and quantification of cross-sectional area of myocytes (middle). Scale bar: 50  $\mu$ m. qPCR analysis of hypertrophic markers ANF and BNP mRNA expression in the hearts (right; n=6 mice per group). (D) Representative Masson's trichrome staining (left) and quantification of collagen deposition in the heart sections of each group (middle; n=6 per group). Scale bar: 100  $\mu$ m. qPCR analysis of collagen I and collagen III mRNA expression in the hearts (right; n=6 per group). (E) Representative H&E and immunohistochemical staining of Mac-2-positive cells (left) and quantification of these cells (middle; n=6 per group). Scale bar: 20  $\mu$ m; qPCR analysis of IL-1 $\beta$  and TNF- $\alpha$  mRNA expression in the hearts (right; n=6 per group). Data are expressed as mean  $\pm$  SEM. \*P < 0.05 vs WT BM to WT.

Figure 5. CD1d deficiency reduces IL-10 expression by NKT cells resulting in fibroblast activation. (A) qPCR analysis of the mRNA level of IL-10 in the hearts of wild type (WT) and CD1dKO mice after Ang II infusion (n=6 per group). (B) qPCR analysis of IL-10 mRNA level in vehicle- or aGC-treated WT hearts after Ang II infusion (n=6 per group). (C) ELISA of the protein level of

IL-10 in WT and CD1dko mice after Ang II infusion (n=6 per group). (D) ELISA of the protein level of IL-10 in WT mice treated with vehicle or aGC after Ang II infusion (n=6 per group). (E) qPCR analysis of IL-10 mRNA level in DCs or NKT cells from WT mice after saline or Ang II treatment. (F) qPCR analysis of IL-10 mRNA level in NKT cells co-cultured with DCs from WT or CD1dko mice after Ang II treatment (n=6 per group). (G) Immunoblotting analysis of the protein levels of  $\alpha$ -SMA, TGF- $\beta$ 1, p-Smad2/3 and Smad2/3 in cardiac fibroblasts (CFs) co-cultured with NKT cells from WT or CD1dko mice after Ang II-infusion (left) and quantification (right; n=5 per group). Data are expressed as mean  $\pm$  SEM. \* $P$  <0.05 vs saline groups; # $P$ <0.05 vs Ang II group.

Figure 6. IL-10 administration reverses cardiac remodeling in CD1d-deficient mice. (A) Echocardiography was performed in wild type (WT) and CD1dko mice 14 days after Ang II infusion and IL-10 administration (50  $\mu$ g/kg) (top). Quantification of EF% and FS% (bottom; n=10 per group). (B) Representative H&E staining of heart sections (top). Scale bar: 2 mm. The ratios of heart weight/body weight (HW/BW) and heart weight/tibial length (HW/TL) (bottom; n=10 per group). (C) Representative wheat germ agglutinin (WGA, Scale bar: 50  $\mu$ m) and Masson's trichrome staining in heart sections (Scale bar: 100  $\mu$ m). (D) Quantification of the cross-sectional area of myocytes and collagen deposition (n=6 per group). (E) Representative immunohistochemical staining of the Mac-2 on the heart sections (left). Scale bar: 50  $\mu$ m. Quantification of Mac-2-positive area (right; n=6 per group). (F) Immunoblotting analysis of the protein expression of p-STA3, STAT3, p-p65, p65, TGF- $\beta$ 1 and  $\alpha$ -SMA (left). Quantification of the protein bands (right; n=5 per group). Data are expressed as mean  $\pm$  SEM. \* $P$ <0.05 vs WT+IgG+Ang II; # $P$ < vs CD1dko+IgG+Ang II; \$  $P$ < vs CD1dko+IL-10+Ang II.

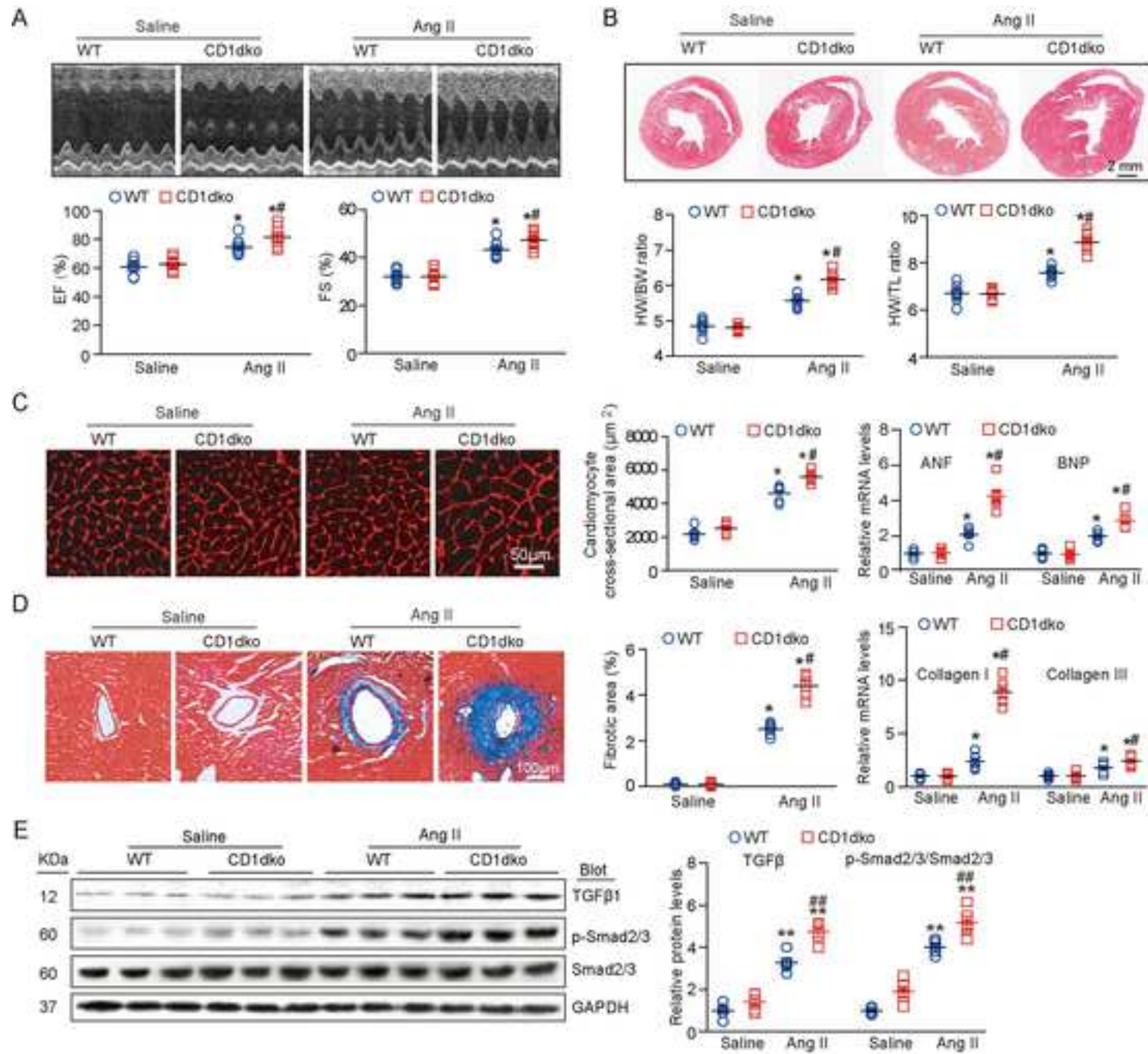


Figure 1

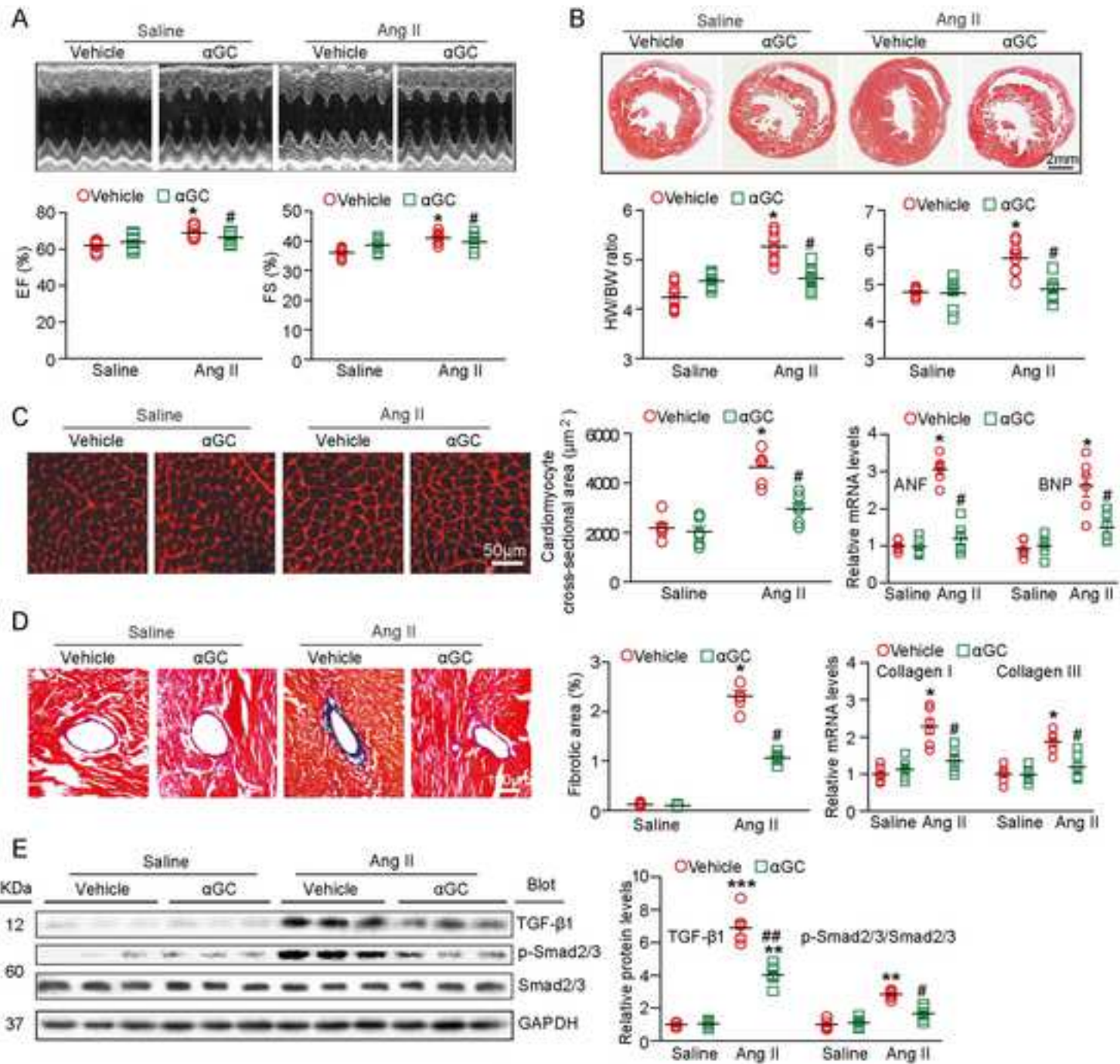


Figure 2

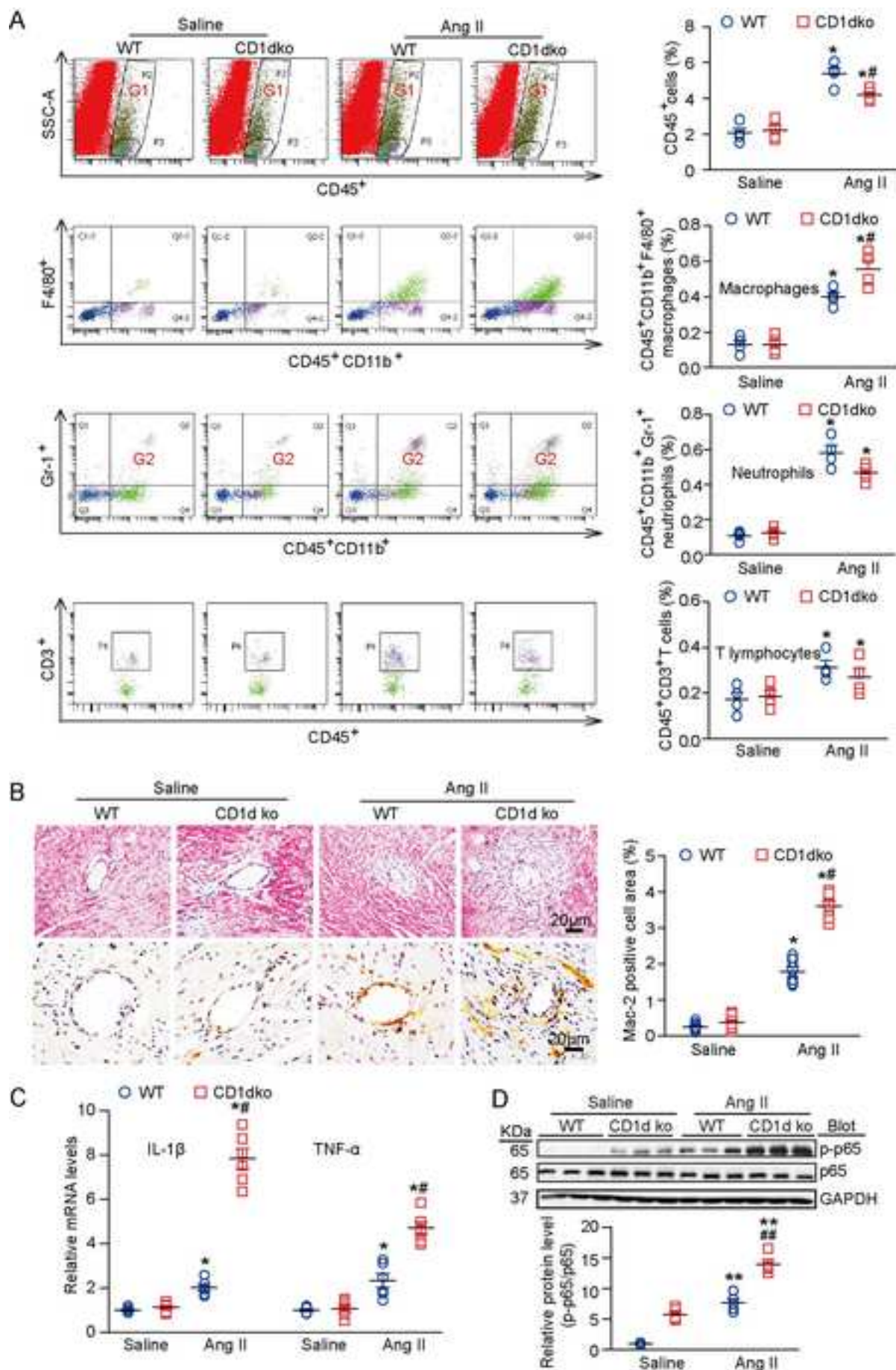


Figure 3

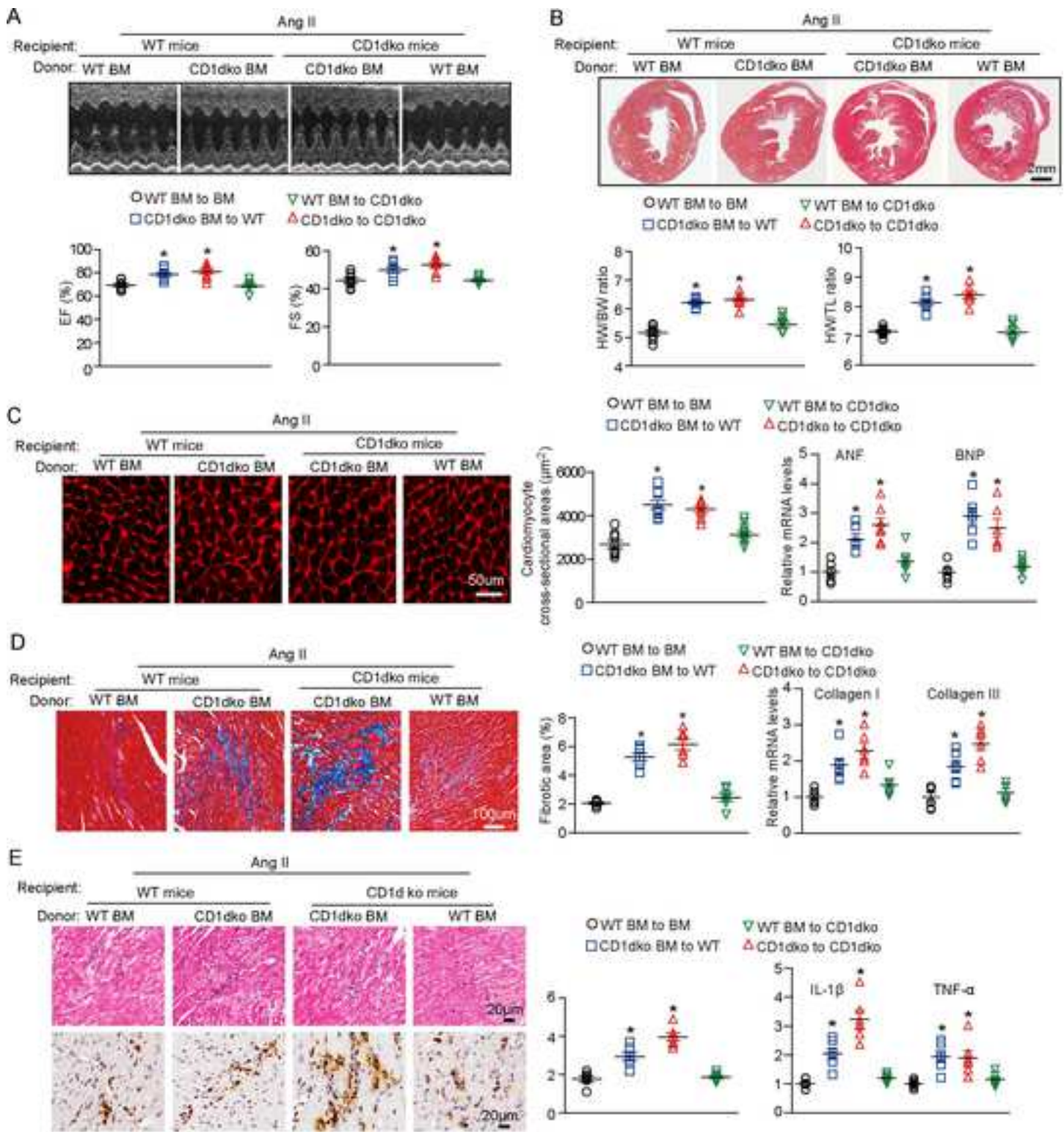


Figure 4

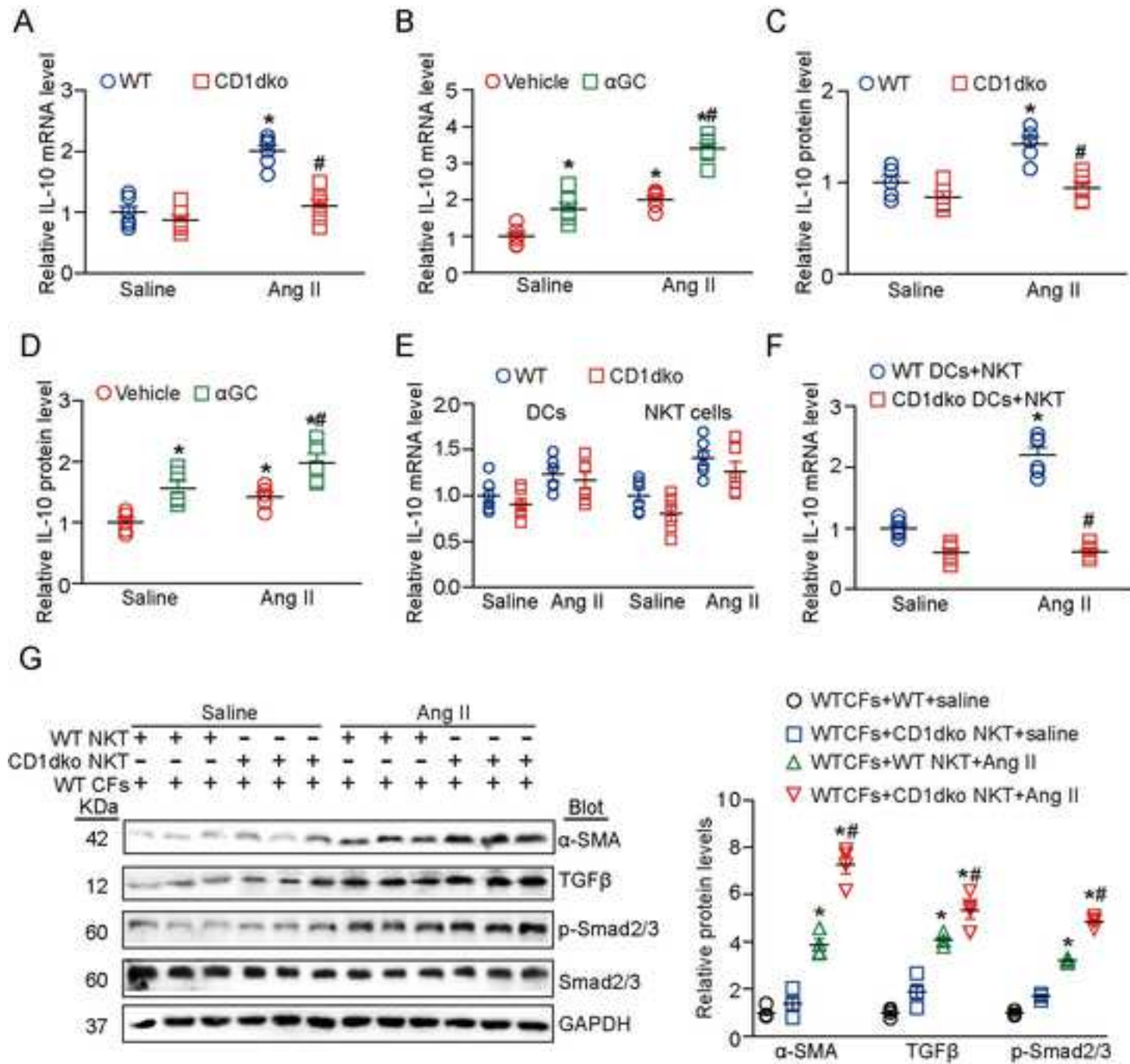


Figure 5

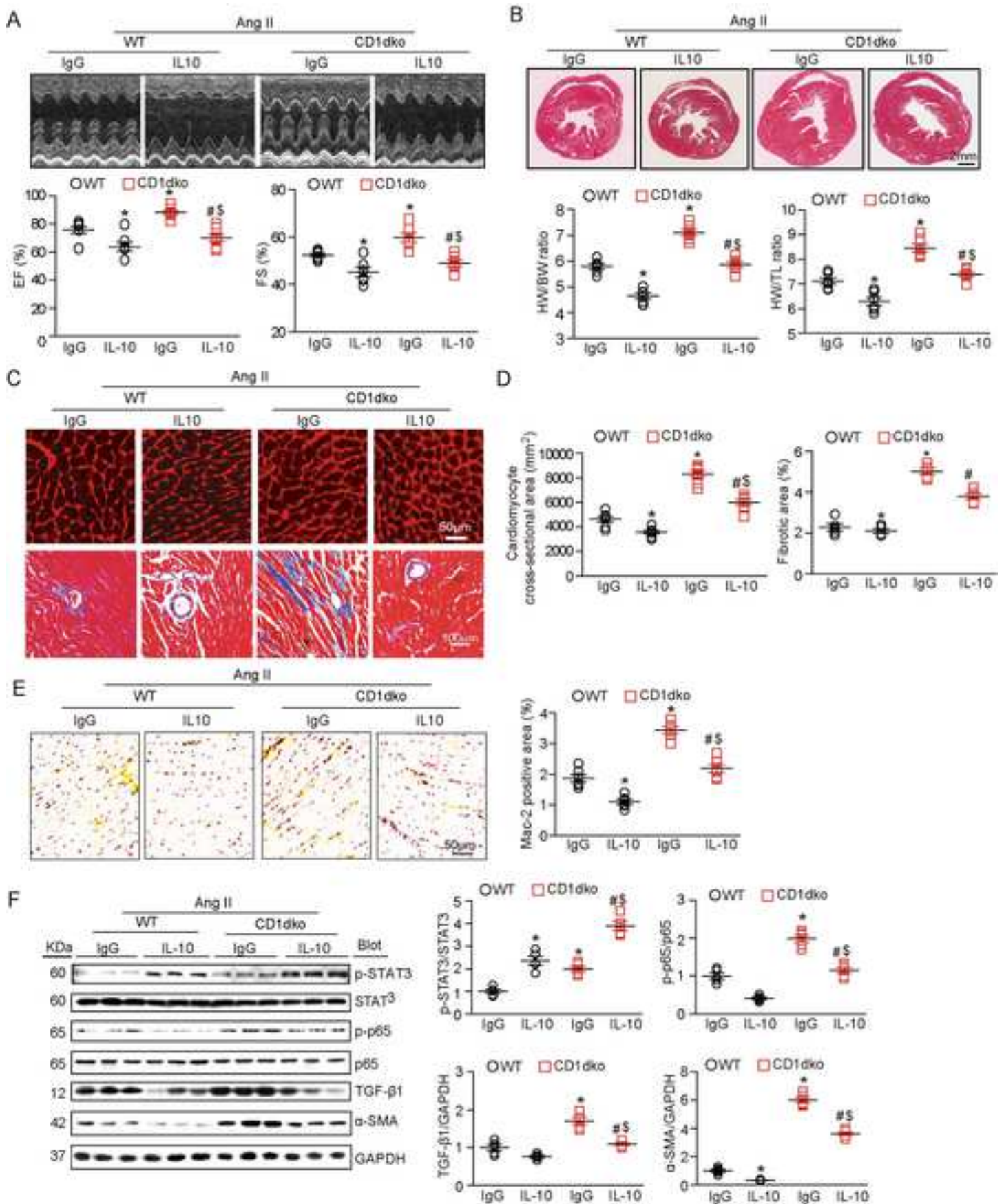


Figure 6